

## Characterization of deltamethrin metabolism by rat plasma and liver microsomes

Sathanandam S. Anand<sup>a,\*</sup>, James V. Bruckner<sup>a</sup>, Wendy T. Haines<sup>c,d,1</sup>, Srinivasa Muralidhara<sup>a</sup>,  
Jeffrey W. Fisher<sup>b</sup>, Stephanie Padilla<sup>c</sup>

<sup>a</sup>Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, University of Georgia, Athens, GA 30602, USA

<sup>b</sup>Department of Environmental Health Science, School of Public Health, University of Georgia, Athens, GA 30602, USA

<sup>c</sup>Neurotoxicology Division, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, USA

<sup>d</sup>Curriculum in Toxicology, UNC-CH, NC 27514, USA

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### Abstract

Deltamethrin, a widely used type II pyrethroid insecticide, is a relatively potent neurotoxicant. While the toxicity has been extensively examined, toxicokinetic studies of deltamethrin and most other pyrethroids are very limited. The aims of this study were to identify, characterize, and assess the relative contributions of esterases and cytochrome P450s (CYP450s) responsible for deltamethrin metabolism by measuring deltamethrin disappearance following incubation of various concentrations (2 to 400  $\mu$ M) in plasma (esterases) and liver microsomes (esterases and CYP450s) prepared from adult male rats. While the carboxylesterase metabolism in plasma and liver was characterized using an inhibitor, tetra isopropyl pyrophosphoramidate (isoOMPA), CYP450 metabolism was characterized using the cofactor, NADPH. Michaelis–Menten rate constants were calculated using linear and nonlinear regression as applicable. The metabolic efficiency of these pathways was estimated by calculating intrinsic clearance ( $V_{\max}/K_m$ ). In plasma, isoOMPA completely inhibited deltamethrin biotransformation at concentrations (2 and 20  $\mu$ M of deltamethrin) that are 2- to 10-fold higher than previously reported peak blood levels in deltamethrin-poisoned rats. For carboxylesterase-mediated deltamethrin metabolism in plasma,  $V_{\max} = 325.3 \pm 53.4$  nmol/h/ml and  $K_m = 165.4 \pm 41.9$   $\mu$ M. Calcium chelation by EGTA did not inhibit deltamethrin metabolism in plasma or liver microsomes, indicating that A-esterases do not metabolize deltamethrin. In liver microsomes, esterase-mediated deltamethrin metabolism was completely inhibited by isoOMPA, confirming the role of carboxylesterases. The rate constants for liver carboxylesterases were  $V_{\max} = 1981.8 \pm 132.3$  nmol/h/g liver and  $K_m = 172.5 \pm 22.5$   $\mu$ M. Liver microsomal CYP450-mediated biotransformation of deltamethrin was a higher capacity ( $V_{\max} = 2611.3 \pm 134.1$  nmol/h/g liver) and higher affinity ( $K_m = 74.9 \pm 5.9$   $\mu$ M) process than carboxylesterase (plasma or liver) detoxification. Genetically engineered individual rat CYP450s (Supersomes) were used to identify specific CYP450 isozyme(s) involved in the deltamethrin metabolism. CYP1A2, CYP1A1, and CYP2C11 in decreasing order of importance quantitatively, metabolized deltamethrin. Intrinsic clearance by liver CYP450s (35.5) was more efficient than that by liver (12.0) or plasma carboxylesterases (2.4).

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### Introduction

Pyrethroid insecticides are used extensively in agriculture and public health (Casida and Quistad, 1998;

Soderlund et al., 2000). As a class, pyrethroid insecticides show high insecticidal potency, tend to exhibit slow development of insect resistance, have relatively low acute toxicity in mammals, and are not persistent in the environment (reviewed in Soderlund et al., 2002). Traditionally, pyrethroid insecticides are divided into two classes (type I and II) based on structure and toxicological actions. Type I compounds do not contain a cyano group, but type II compounds do. Tremor is the major sign of

\* Corresponding author. Fax: +1 706 542 5358.

E-mail address: [sanand@rx.uga.edu](mailto:sanand@rx.uga.edu) (S.S. Anand).

<sup>1</sup> Present address: Lineberger Comprehensive Cancer Center, 102 Mason Farm Road, Campus Box 7295 UNC, Chapel Hill, NC 27599, USA.

poisoning by type I pyrethroid insecticides, while choreoathetosis and salivation are the major signs of type II poisoning (Glickman and Casida, 1982; Lawrence and Casida, 1982). Deltamethrin ((*S*)- $\alpha$ -cyano-3-phenoxybenzyl-(1*R*,*cis*)-2,2-dimethyl-3-(2,2-dibromovinyl)-cyclopropanecarboxylate) is a type II pyrethroid and is considered the most potent neurotoxic pyrethroid (Pham et al., 1984; Ruzo and Casida, 1977). Unlike most pyrethroid insecticides that exist as at least two isomers, deltamethrin is marketed as a single isomer (*cis*) (Elliott et al., 1974) and is widely used in veterinary products, as well as in agricultural formulations (ATSDR, 2003; CALEPA, 2001; Soderlund et al., 2000).

The acute toxicity, as well as cellular and molecular mechanisms of toxic action of deltamethrin, has been studied extensively (Narahashi, 1985, 1986). Following acute oral administration of 15 or 50 mg/kg to rats, profuse salivation, coarse body tremors, biting or gnawing, lacrimation, choreoathetosis, and mortality were observed (Soderlund et al., 2000). The marked neurotoxic effects of deltamethrin have been attributed to its binding to sodium channels in the brain (Narahashi, 1982, 1985; Tabarean and Narahashi, 2001) and to interaction with the GABA receptor–ionophore complex (Bloomquist et al., 1986; Crofton and Reiter, 1987; Lawrence and Casida, 1983). The parent compound is considered to be the primary neurotoxicant (Rickard and Brodie, 1985), but the toxicity of the major oxidative metabolite, 4'-OH deltamethrin (Fig. 1), is in question (Anadon et al., 1996; Dayal et al., 2003;

Gray and Rickard, 1982). Other metabolites such as 4'-OH-phenoxybenzoic acid, phenoxybenzoic acid, and thiocyanate elicit toxicity at concentrations that are not achieved from deltamethrin metabolism in vivo (NRCC, 1986).

While the toxicity of deltamethrin has been extensively characterized, toxicokinetic data for deltamethrin and most other pyrethroids are scant and incomplete. Deltamethrin is rapidly absorbed when administered orally or intraperitoneally and enters the central and peripheral nervous systems (Anadon et al., 1996; Rickard and Brodie, 1985). Deltamethrin is metabolized and its metabolites excreted over a period of days in rats. Anadon et al. (1996) reported the elimination half-life to be 38.5 h after an oral dosage of 26 mg/kg, while Ruzo et al. (1978) found that deltamethrin and its metabolites were largely excreted within 4 days of oral administration of 0.6–1.6 mg/kg. Ester cleavage and oxidation, primarily at the 4' position, are the two major means of deltamethrin metabolism (Fig. 1) (Ruzo et al., 1978, 1979; Soderlund and Casida, 1977). Esterases catalyze hydrolysis of the ester bond to form relatively non-toxic acid and alcohol moieties, whereas CYP450s catalyze aromatic hydroxylation of deltamethrin at various positions, notably the 4' position, followed by conjugation (Ruzo and Casida, 1977; Ruzo et al., 1978, 1979; Shono et al., 1979; Soderlund and Casida, 1977). Ruzo et al. (1978) characterized the metabolites of ester cleavage and CYP450 oxidation, but the metabolic rate constants and relative contribution as assessed by intrinsic clearance of these pathways are unknown.

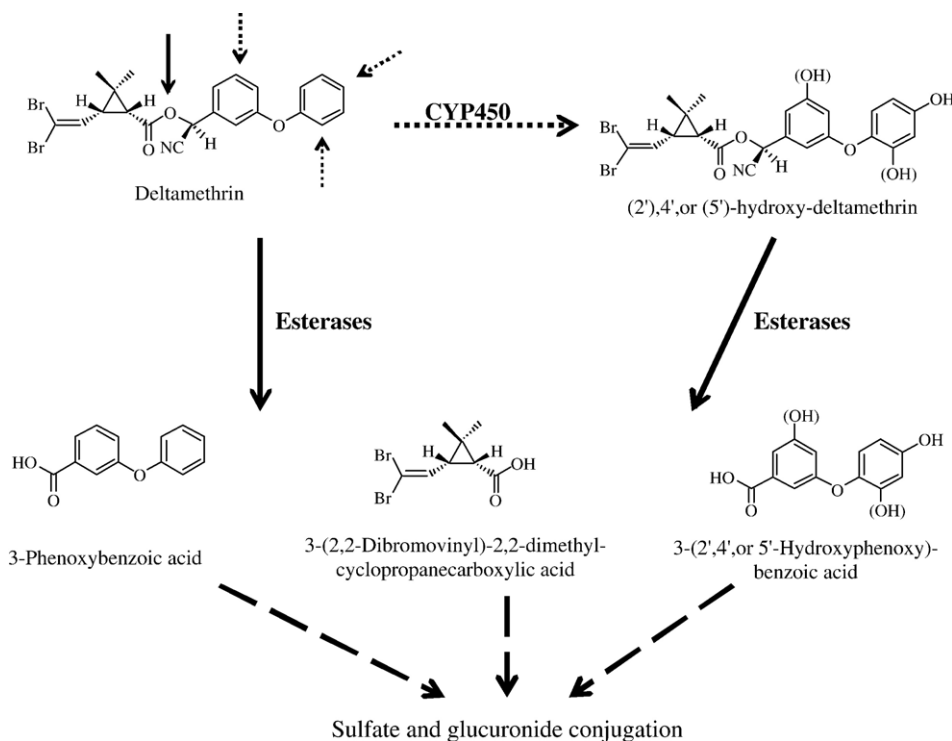


Fig. 1. Metabolism of deltamethrin. It is generally accepted that deltamethrin is detoxified by CYP450-mediated oxidation (dotted arrows) and esterase-mediated hydrolysis (solid arrows) followed by conjugation.

Although organophosphates that inhibit carboxylesterase activity are shown to increase deltamethrin toxicity in vivo (Ruzo et al., 1977) and decrease metabolism in vitro (Ruzo et al., 1979), there has been little attention paid to identification of the esterases that cleave deltamethrin or other pyrethroid insecticides. Recently, Stok et al. (2004) isolated and characterized two pyrethroid-hydrolyzing carboxylesterases from mouse liver microsomes. The one tested showed very little capacity to hydrolyze deltamethrin. In addition, Satoh et al. (1999) have proposed that A-esterases metabolize pyrethroid insecticides. The identity of CYP450 isozymes responsible for deltamethrin hydroxylation has not been established, although CYP1A1/2 and CYP2B1/2 may be involved as they are induced after subacute deltamethrin administration (Dayal et al., 1999).

The objectives of the present study were to identify, characterize, and assess the relative contributions of the enzyme(s) responsible for deltamethrin metabolism in adult rat liver and plasma by deriving metabolic rate constants for the esterases (plasma and liver microsomes) and CYP450s (liver microsomes). This information is being used in construction of a physiologically based pharmacokinetic (PBPK) model for deltamethrin in the adult male rat.

## Materials and methods

**Chemicals.** Deltamethrin ((*S*)- $\alpha$ -cyano-3-phenoxybenzyl-(1*R*,*cis*)-2,2-dimethyl-3-(2,2-dibromovinyl)-cyclopropanecarboxylate) was obtained from ChemService (Westchester, PA, 98% pure). Acetone, methanol (HPLC-grade), and acetonitrile (HPLC-grade) were procured from Burdick and Jackson (Muskegon, MI). Tetra isopropyl pyrophosphoramidate (isoOMPA) was purchased from Sigma Chemical Co (St. Louis, MO). All other chemicals were of analytical grade. Phenyl saligenin phosphate (PSP) was a generous gift from Dr. Marion Ehrich (Division of Pharmacology and Toxicology, VA/MD Regional College of Veterinary Medicine, Blacksburg, VA).

**Animals.** Male Sprague–Dawley (S–D) rats (90 days old, 350–400 g) were obtained from Charles River Inc. (Raleigh, NC). Each rat was housed individually on ALPHA-dri (virgin cellulose paper product; Shepherd Specialty Papers, Watertown, TN) bedding and allowed food (Purina rat chow) and water ad libitum. The AAALAC-accredited animal facility (EPA, Research Triangle Park, NC) was maintained at  $72 \pm 2$  °F,  $50 \pm 10\%$  humidity, with a 12-h light:dark cycle. All animal handling procedures followed NIH guidelines and were approved by the US EPA National Health and Environmental Effects Research Laboratory Animal Care and Use Committee.

**Plasma and microsome preparation.** Although esterases and CYP450s are present in many tissues, liver microsomes

and plasma were chosen to study deltamethrin metabolism. Liver is the major metabolic organ and has the highest levels of carboxylesterase activities (Satoh and Hosokawa, 1998) and CYP450s. Plasma carboxylesterases are generally considered the first quantitatively important enzymes that orally administered xenobiotics with an ester linkage encounter. Plasma and liver samples were collected from untreated rats anesthetized with carbon dioxide. The animals were decapitated and trunk blood collected in heparinized centrifuge tubes. The blood samples were centrifuged at  $2500 \times g$  for 10 min in a Sorvall MC 12 V microcentrifuge (DuPont, Atlanta, GA) to obtain plasma. The plasma was frozen at  $-80$  °C until use.

To prepare hepatic microsomes by the procedure of Omura and Sato (1964), 5 g of fresh liver was homogenized in 25 ml of cold 0.02 M Tris–KCl buffer, pH 7.4, using a Teflon/glass tissue homogenizer. Briefly, the homogenized liver was centrifuged using a Sorvall RC5C centrifuge (DuPont, Newton, CT) for 30 min at 4 °C at  $12,000 \times g$ . The supernatant was collected and centrifuged using a Beckman Coulter Optima XL-100K ultracentrifuge (Fullerton, CA) at 4 °C at  $105,000 \times g$  for 60 min. The pellet containing the microsomes was re-suspended in Tris–KCl, pH 7.4 buffer and frozen at  $-80$  °C. Protein content of the microsomes was measured by the method of Lowry et al. (1951). Since the liver microsomes were re-suspended in a volume of buffer the same as the original volume of the liver, enzyme activities measured in each ml of suspension could be expressed per gram of liver.

**Carboxylesterase assay.** Carboxylesterase activity was determined using a microtiter plate-based assay (Chanda et al., 1997). Microsomes [20  $\mu$ l of 1:600 (vol:vol) dilution] or plasma (10  $\mu$ l of 1:10) was mixed with 20  $\mu$ l EGTA (1 mM final) and brought up to 100  $\mu$ l using Tris buffer (50 mM, pH 8). To this mixture, 100  $\mu$ l of the substrate, *p*-nitrophenol acetate (*p*NPA; 0.88 mM final concentration), was added to initiate the reaction. Carboxylesterase activity was measured at 405 nm by reading the generation of *p*-nitrophenol using a spectrophotometer (ThermoMax Microtiter Plate Reader, Molecular Devices, Menlo Park, CA). A standard curve of *p*-nitrophenol was constructed by adding increasing amounts of *p*-nitrophenol (Chanda et al., 1997).

**Carboxylesterase inhibition.** Two well-known carboxylesterases inhibitors, phenyl saligenin phosphate (PSP) in methanol and isoOMPA in water (Chanda et al., 1997), were used in the present study to help identify the role of carboxylesterases in deltamethrin metabolism. The efficacy of these two inhibitors was measured by incubating 5  $\mu$ l PSP (500 nM final concentration for plasma and 100 nM final concentration for liver microsomes) or 10  $\mu$ l isoOMPA (1 mM final concentration for plasma and 500  $\mu$ M final concentration for liver microsomes) for specified times: 15 to 120 min for liver microsomes and 60 and 120 min for

plasma. After preincubation with the inhibitors, carboxylesterase activity was measured as described above. Both inhibitors showed equal efficacy (Fig. 2), but isoOMPA was chosen for further studies as it is water-soluble.

**Metabolism of deltamethrin.** The kinetics of in vitro metabolism of deltamethrin in plasma and microsomes was evaluated by measuring the disappearance of parent compound as opposed to measuring the generation of metabolites. Hydroxylated metabolites of deltamethrin are not commercially available to use as standards. Initial experiments were conducted to establish the optimal incubation conditions for each assay (plasma carboxylesterases, liver carboxylesterases, and liver CYP450s): disappearance of deltamethrin was linear with respect to time as well as to microsome or plasma levels over concentration ranges relevant to this study. Note that separate incubations were performed for carboxylesterase (30-min incubation for plasma or liver microsomes) and CYP450 (10-min incubation) metabolism. The velocity ( $V_o$ ) of the reaction was expressed as nmol deltamethrin disappearance/h/g liver or ml plasma.

**Plasma metabolism of deltamethrin.** Various concentrations (2, 20, 100, and 200  $\mu$ M final concentration) of deltamethrin were incubated with 20% plasma (diluted using Tris–HCl buffer, pH 7.4) for 30 min at 37 °C. One set of plasma samples was preincubated with isoOMPA for 1 h before adding deltamethrin. The reaction was initiated by adding 1 ml of 20% plasma (with or without isoOMPA) to 10  $\mu$ l of various deltamethrin concentrations (in acetone, never more than 1% of total volume). Similar incubations were conducted with Tris–HCl buffer, which

served as a control for non-enzymatic hydrolysis of deltamethrin. After 30 min of incubation at 37 °C, 4 ml of acetone was added to stop the reaction and to extract the deltamethrin. The difference between the reaction tubes that did and did not contain isoOMPA was considered as carboxylesterase-mediated metabolism. The total metabolism was calculated from the difference between the buffer background tube and the tube without isoOMPA.

**A-esterase involvement in deltamethrin metabolism.** As isoOMPA only partially inhibited plasma deltamethrin metabolism at higher deltamethrin concentrations (100 and 200  $\mu$ M), experiments were conducted to determine whether A-esterases could metabolize deltamethrin as reported by Satoh et al. (1999). Ten microliters of deltamethrin (final concentrations of 100 and 200  $\mu$ M) were incubated with 1 ml of 20% plasma with and without EGTA (1 mM, final) for 30 min at 37 °C. EGTA inhibits A-esterase activity by  $\text{Ca}^{2+}$  chelation. A buffer control was included to account for background hydrolysis. After 30 min, 4 ml of acetone was added for deltamethrin extraction. The difference between ‘with’ and ‘without’ EGTA was considered to be due to A-esterase-mediated metabolism.

**Role of butyrylcholinesterase in deltamethrin metabolism.** Since isoOMPA is a known inhibitor of butyrylcholinesterase activity, the role of this enzyme in the metabolism of deltamethrin was examined using purified horse serum butyrylcholinesterase (1000 U, Sigma Chemical Co, St. Louis, MO). Butyrylcholinesterase solution was prepared in Tris buffer (50 mM, pH 7.4) to yield a final concentration of 1 U/ml. The enzyme and heat-inactivated enzyme preparations (control) were incubated in duplicate with 2 or 100  $\mu$ M deltamethrin at 37 °C. After 30 min, 4 ml of acetone was added for deltamethrin extraction. The difference between ‘enzyme’ and ‘heat-inactivated enzyme’ was considered to be due to butyrylcholinesterase-mediated metabolism.

## Microsomal metabolism of deltamethrin

### Carboxylesterases

Microsomes [1:25 vol/vol (~0.5 mg protein/ml) with Tris–HCl buffer, pH 7.4] were preincubated with or without isoOMPA for 1 h at room temperature. One milliliter of microsomes with or without isoOMPA was added to 10  $\mu$ l of various concentrations of deltamethrin to initiate the reaction. A buffer control was included to account for background hydrolysis. After 30 min of incubation at 37 °C, 4 ml of acetone was added to the incubation mixture to stop the reaction and to extract the deltamethrin. The difference between ‘with’ and ‘without’ isoOMPA was considered as carboxylesterase-mediated metabolism.

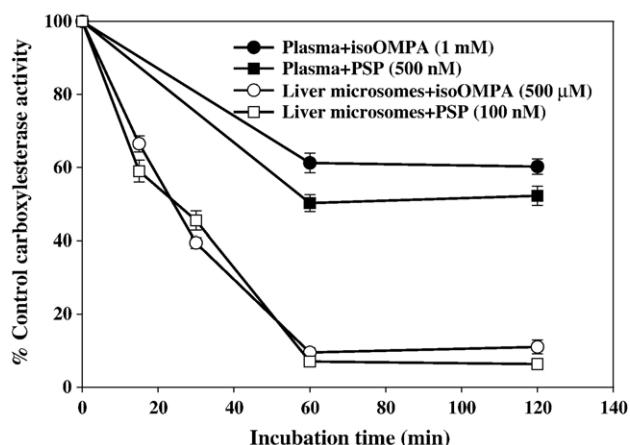


Fig. 2. Inhibition of plasma and liver microsomal carboxylesterase activity. Hepatic microsomes or plasma was incubated with the indicated concentrations of isoOMPA and PSP for various durations at 26 °C. Carboxylesterases activity was assessed using a microtiter-based method (see Materials and methods). Data represent percentages of control values. Control activity for the plasma =  $1.5 \pm 0.0$   $\mu$ mol *p*NP produced/min/ml and for hepatic microsomes =  $25.7 \pm 1.4$   $\mu$ M *p*NP produced/min/mg microsomal protein (mean  $\pm$  SE,  $n = 4$ ).

### Cytochrome P450s

Deltamethrin metabolism by CYP450s was assessed by incubating 1 ml of microsomes (microsomes were diluted to get 0.5 mg protein/ml using Tris–KCl buffer pH 7.4) with 10  $\mu$ l of a series of deltamethrin concentrations (2 to 200  $\mu$ M final) with and without NADPH (1.2 mM, final) at 37 °C for 10 min. The reaction was initiated by adding microsomes. A buffer control was included to account for non-enzymatic hydrolysis. After 10 min of incubation, 4 ml of acetone was added to the incubation mixture to terminate the reaction and extract the deltamethrin. The difference between ‘with’ and ‘without’ NADPH was considered as CYP450-mediated metabolism.

### Extraction

Upon addition of acetone, the tubes were vortexed for 5 min and centrifuged for 10 min at 2500  $\times$  g (HNS-centrifuge, Damon, Needham Heights, MA). The supernatant was transferred to another centrifuge tube, and the pellet washed with another 4 ml of acetone. The supernatants were combined and evaporated to dryness under a stream of nitrogen at room temperature. After complete drying, the residue was reconstituted in 200  $\mu$ l methanol, vortexed, and passed through a GHP Acrodisc syringe filter (13 mm diameter, 0.45  $\mu$ m pore size, Waters, MA) to remove the suspended solids before transferring to a HPLC sample vial (Haines et al., 2004).

### HPLC assay of deltamethrin

The deltamethrin concentration in plasma and liver microsomes was measured according to the method of Haines et al. (2004). The HPLC system used was manufactured by Waters (Milford, MA) and consisted of a 2690 Separations Module and a 996 PDA detector. The column used was a Waters Spherisorb 5- $\mu$ m C8 column (Waters, MA, 150 mm long  $\times$  4.6 mm internal diameter) and a Waters Spherisorb S5 C8 guard column (10 mm long  $\times$  4.6 mm internal diameter). Different mobile phases, 70:30 acetonitrile/1% sulfuric acid at a flow rate of 1 ml/min for microsomes and 80:20 acetonitrile/1% sulfuric acid at a flow rate of 0.5 ml/min for plasma, were employed to reduce the background in the liver microsomes. The volume of sample injected was 20  $\mu$ l. Duplicate injections were made of all samples and standards, with the average of the two injections used for calculation of the deltamethrin concentration. Peak heights were quantified at 230 nm by the external standard technique using standard solutions of deltamethrin (5 ng to 10  $\mu$ g). Diphenylamine was used as an internal standard.

### Identification of CYP450 isozymes responsible for deltamethrin metabolism

In vitro studies were conducted with 14 cDNA-expressed rat CYP isoforms (Supersomes) to identify the isozyme(s)

capable of metabolizing deltamethrin. Microsomes from baculovirus-infected insect cells (BTI-TN-5B1-4) expressing CYP1A1, CYP1A2, CYP2A1, CYP2A2, CYP2B1, CYP2C6, CYP2C11, CYP2C12, CYP2C13, CYP2D1, CYP2E1, CYP2D2, CYP3A1, and CYP3A2 were obtained from Gentest, a BD Sciences company (Woburn, MA). The isoforms, CYP2A1, CYP2A2, CYP2B1, CYP2C6, CYP2C11, CYP2C12, CYP2C13, CYP2E1, CYP3A1, and CYP3A2 were co-expressed with CYP450 reductase and cytochrome b5. CYP1A1, CYP1A2, CYP2D1, and CYP2D2, however, were co-expressed with CYP450 reductase only. Since the objective of these studies was to establish the identity of CYP450 isozymes that can metabolize deltamethrin, the metabolism was measured by incubating a single high concentration of deltamethrin (200  $\mu$ M;  $\sim$ 2.5 times the CYP450  $K_m$ ) with 50 pmol CYP450 of each isozyme with or without NADPH (1.2 mM) for 15 min. The corresponding dilution of each isozyme was made using Tris–HCl buffer, pH 7.4. The reaction was initiated by adding NADPH. Controls were performed with insect microsomal system and buffer. Deltamethrin was quantified by the HPLC method described above.

### Data analyses and statistics

Microsomal and plasma incubations were conducted with  $n = 4$  samples collected from individual animals. Means and standard error (SE) were calculated. The Michaelis–Menten metabolic rate constants ( $V_{max}$  and  $K_m$ ) for deltamethrin disappearance were calculated by nonlinear regression for carboxylesterase metabolism (plasma and liver) and CYP450 metabolism (liver) and by linear regression for total metabolism in plasma using Prism (3.03). The data were subjected to one-way ANOVA followed by Tukey’s Studentized range (HSD) test using SAS Version 8.02 (SAS Institute Inc., Cary, NC).

## Results

### Characteristics of hepatic microsomal and plasma carboxylesterases inhibition

The microtiter plate assay was used to determine the most appropriate inhibitor concentration and duration of preincubation for maximal inhibition of both hepatic microsomal and plasma carboxylesterases to characterize deltamethrin metabolism. Initially, the time-course of inhibition was assessed for both microsomal and plasma carboxylesterases over time using two different inhibitors. The concentration of inhibitors (isoOMPA-1 mM for plasma and 500  $\mu$ M for microsomes and PSP-500 nM for plasma and 100 nM for microsomes) for deltamethrin metabolism was chosen from preliminary experiments, in which these concentrations elicited maximum inhibition of carboxylesterase activity as assessed using the microtiter plate assay.

As can be seen in Fig. 2, both plasma and liver microsomal carboxylesterase inhibition were maximal after 60 min of incubation with either inhibitor. isoOMPA and PSP were equipotent in inhibiting plasma and microsomal carboxylesterase activity (as measured by *p*NPA hydrolysis) 50% and 90%, respectively. Chlorpyrifos-oxon (3  $\mu$ M) and profenofos (10  $\mu$ M) also inhibited 50% of carboxylesterase activity in plasma (data not shown). IsoOMPA was chosen for subsequent studies because it is water-soluble. The conditions for maximal carboxylesterase inhibition by isoOMPA (for plasma, 1 mM; for liver microsomes, 500 nM; 1 h preincubation) were used in the assays of plasma or microsomal metabolism of deltamethrin. Therefore, carboxylesterase-catalyzed metabolism of deltamethrin was defined by the portion of the metabolism that was sensitive to isoOMPA inhibition.

#### Plasma metabolism of deltamethrin

The rate of deltamethrin metabolism was taken to be the rate of deltamethrin disappearance from plasma in the presence or absence of isoOMPA (Fig. 3). Interestingly, there were two distinct concentration-dependent components of deltamethrin metabolism in rat plasma: an isoOMPA-sensitive component (carboxylesterases) and a non-isoOMPA-sensitive component. At the lower deltamethrin concentrations (2 and 20  $\mu$ M), isoOMPA completely inhibited plasma metabolism of delta-

methrin, but at the higher concentrations (100 and 200  $\mu$ M), the inhibition was only partial. Subtracting 'with isoOMPA' from 'without isoOMPA' showed that the rate of isoOMPA-sensitive carboxylesterase metabolism was linear at low deltamethrin concentrations and plateaued at higher deltamethrin concentrations (Fig. 3, insert B). The total plasma metabolism of deltamethrin (without isoOMPA, i.e., isoOMPA-sensitive + non-isoOMPA-sensitive) (Fig. 3) did not appear to plateau at any deltamethrin concentration used. Metabolic rate constants were calculated for total metabolism using linear regression (Lineweaver–Burk plot,  $1/V_o$  vs.  $1/S$ ) (Fig. 3, insert A) and for isoOMPA-sensitive metabolism using nonlinear regression (Fig. 3, insert B). The apparent  $V_{max}$  and  $K_m$  for total metabolism of deltamethrin in the plasma were  $1720.8 \pm 53.1$  nmol/h/ml and  $1138.8 \pm 38.3$   $\mu$ M, respectively, whereas the carboxylesterase-mediated metabolism of deltamethrin in the plasma exhibited a much lower  $V_{max}$  and  $K_m$ :  $325.3 \pm 53.4$  nmol/h/ml and  $165.4 \pm 41.9$   $\mu$ M, respectively.

#### Role of A-esterases in deltamethrin metabolism

The rate of deltamethrin disappearance in plasma with or without the addition of EGTA was not different (Fig. 4). This indicates that plasma A-esterases are not involved in deltamethrin metabolism. Furthermore, the presence of EGTA did not alter deltamethrin disappearance in liver microsomes (data not shown).

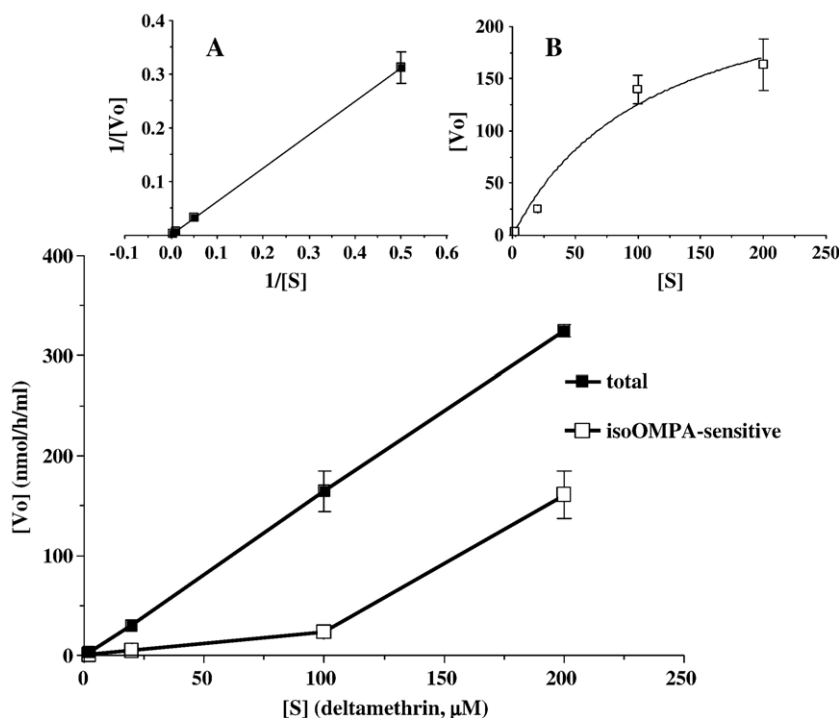


Fig. 3. Rate of deltamethrin metabolism in plasma. Various concentrations of deltamethrin were incubated with 20% plasma with or without isoOMPA preincubation (1 h) for 30 min at 37 °C. Deltamethrin metabolism was measured by disappearance of deltamethrin. Values represent mean  $\pm$  SE for 4 plasma samples from 4 individual animals. Rate of the reaction without isoOMPA was considered as total metabolism, whereas the rate of the reaction with isoOMPA was considered as carboxylesterase metabolism. Insert A represents Lineweaver–Burk plot,  $y$ -intercept =  $1/V_{max}$ ,  $x$ -intercept =  $-1/K_m$ , for the total metabolism ( $V_{max}$ — $1720.8 \pm 53.1$  nmol/h/ml;  $K_m$ — $1138.8 \pm 38.3$   $\mu$ M). Correlation coefficient ( $r^2$ ) is 0.99. Insert B represents nonlinear regression for carboxylesterases-mediated metabolism ( $V_{max}$ — $325.3 \pm 53.4$  nmol/h/ml;  $K_m$ — $165.4 \pm 41.9$   $\mu$ M).

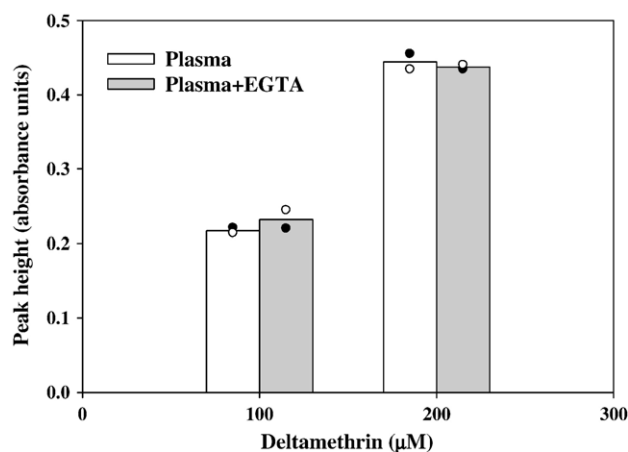


Fig. 4. Lack of deltamethrin metabolism by A-esterases. Two concentrations (100 and 200  $\mu\text{M}$ ) of deltamethrin were incubated with plasma ( $n = 2$ ) with and without EGTA (1 mM). Details of incubation conditions are the same as in Fig. 2. Addition of EGTA did not alter deltamethrin disappearance, indicating that A-esterases are not responsible for deltamethrin metabolism. Symbols represent individual values, whereas the bar height is the mean of the two values.

#### Role of butyrylcholinesterase in deltamethrin metabolism

Deltamethrin disappearance was not different between pure and heat-inactivated enzyme (data not shown). The data rule out the possible involvement of butyrylcholinesterase in deltamethrin metabolism.

#### Hepatic microsomal metabolism of deltamethrin

The rate of deltamethrin metabolism by liver microsomal carboxylesterases (Fig. 5) was dependent upon the substrate concentration up to 100  $\mu\text{M}$  and appeared to be approaching

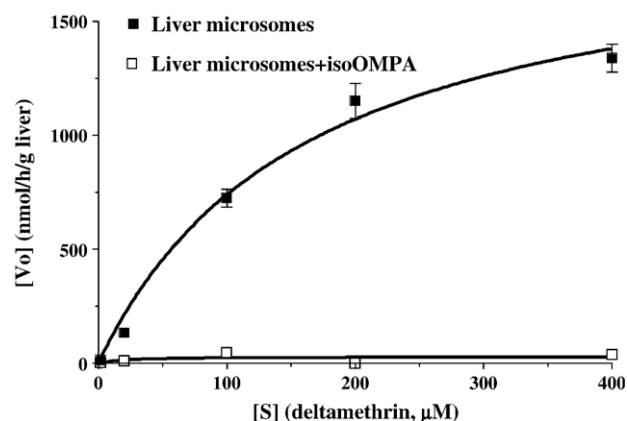


Fig. 5. Rate of deltamethrin metabolism by liver microsomal carboxylesterases. A series of concentrations of deltamethrin were incubated with liver microsomes with or without isoOMPA preincubation for 1 h at 37  $^{\circ}\text{C}$ . Details of the incubation conditions are given in Materials and methods. Values represent mean  $\pm$  SE for 4 hepatic microsomal samples from individual animals. Michaelis–Menten rate constants were calculated by nonlinear regression,  $V_{\text{max}} = 1981.8 \pm 132.3$  nmol/h/g liver;  $K_m = 172.5 \pm 22.5$   $\mu\text{M}$ .

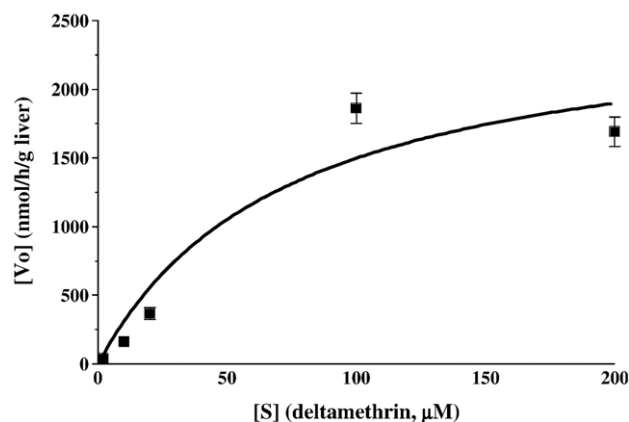


Fig. 6. Rate of deltamethrin metabolism by liver microsomal CYP450s. Various concentrations of deltamethrin were incubated with liver microsomes (0.5 mg protein/ml) with or without NADPH (1.2 mM) for 10 min at 37  $^{\circ}\text{C}$ . Details of incubation conditions are given in Materials and methods. Values represent mean  $\pm$  SE for 4 independent microsomal samples from 4 different animals. Michaelis–Menten rate constants were calculated by nonlinear regression,  $V_{\text{max}} = 2611.3 \pm 134.1$  nmol/h/g liver;  $K_m = 74.9 \pm 5.9$   $\mu\text{M}$ .

saturation by 200 to 400  $\mu\text{M}$ . Unlike plasma, the addition of isoOMPA completely inhibited deltamethrin metabolism at all the deltamethrin concentrations studied. The Michaelis–Menten rate constants for the microsomal carboxylesterase-mediated metabolism of deltamethrin were calculated using nonlinear regression,  $V_{\text{max}} = 1981.8 \pm 132.3$  nmol/h/g liver and  $K_m = 172.5 \pm 22.5$   $\mu\text{M}$ .

Fig. 6 presents deltamethrin metabolism by liver microsomal CYP450s. The initial velocity of deltamethrin disappearance was dependent upon substrate concentration up to 100  $\mu\text{M}$ , approaching substrate saturation by 200  $\mu\text{M}$ . The Michaelis–Menten rate constants for the microsomal CYP450 metabolism of deltamethrin were calculated using

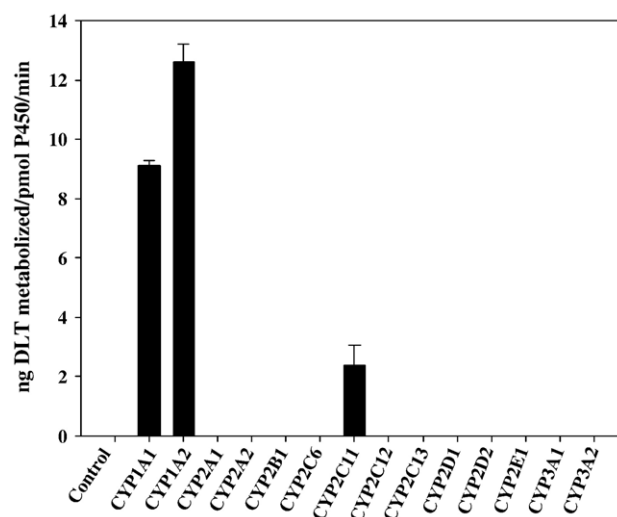


Fig. 7. Deltamethrin metabolism by individual rat CYP450 isoform Supersomes. Two hundred micromolar deltamethrin was incubated with each isoform for 15 min at 37  $^{\circ}\text{C}$  with and without NADPH. Values represent an  $n = 1$  observation with the mean  $\pm$  SE for 3 injections.

Table 1

Metabolic rate constants and intrinsic clearance of deltamethrin by plasma and liver microsomal carboxylesterases and liver microsomal CYP450s

Enzyme		Vmax	Km	Intrinsic clearance (Vmax/Km)
Plasma	Total metabolism	1720.8 ± 53.1 <sup>b</sup>	1138.8 ± 38.3 <sup>c</sup>	1.6 ± 0.1 <sup>a</sup>
	carboxylesterase metabolism	325.3 ± 53.4 <sup>c</sup>	165.4 ± 41.9 <sup>ab</sup>	2.4 ± 0.4 <sup>a</sup>
Liver microsomes	carboxylesterase metabolism	1981.8 ± 132.3 <sup>b</sup>	172.5 ± 22.5 <sup>b</sup>	12.0 ± 1.1 <sup>b</sup>
	CYP450 metabolism	2611.3 ± 134.1 <sup>a</sup>	74.9 ± 5.9 <sup>a</sup>	35.5 ± 2.9 <sup>c</sup>

Results are expressed as mean ± SE ( $n = 4$ ). The data with common superscripts do not differ significantly from each other ( $P < 0.05$ ). Vmax values are expressed as nmol/h/g liver or ml; Km values are expressed as  $\mu\text{M}$ ; Vmax/Km values are expressed as  $\text{h}^{-1}$ .

nonlinear regression,  $V_{\text{max}} = 2611.3 \pm 134.1$  nmol/h/g liver and  $K_{\text{m}} = 74.9 \pm 5.9$   $\mu\text{M}$ .

#### *In vitro* incubation with cDNA-expressed rat CYP450s

Deltamethrin metabolism by cDNA-expressed rat CYP450 isozymes is shown in Fig. 7. CYP1A1, CYP1A2, and CYP2C11 metabolized deltamethrin in the following order: 1A2 > 1A1 > 2C11.

Carboxylesterase and CYP450 metabolic rate constants as well as plasma and liver microsomal intrinsic clearance values are summarized in Table 1. While Vmax values for hepatic microsomal carboxylesterases and total plasma metabolism were similar, the Vmax value for microsomal carboxylesterases was significantly higher than that for plasma carboxylesterase-mediated metabolism and lower than that for CYP450-mediated metabolism. On the other hand, the affinity of the CYP450s for deltamethrin was higher than that of the microsomal carboxylesterases or total plasma metabolism, but similar to plasma carboxylesterases. The intrinsic clearance ( $V_{\text{max}}/K_{\text{m}}$ ) by CYP450s was greater than that by the other pathways. Intrinsic clearance by liver microsomal carboxylesterases was higher than total or carboxylesterase-mediated metabolism in plasma.

## Discussion

Findings in the present study indicate that deltamethrin biotransformation by liver microsomal CYP450s is 3 to 15 times higher than that by either liver or plasma carboxylesterases. Moreover, deltamethrin metabolism by liver carboxylesterases is more efficient than metabolism by plasma carboxylesterases.

It has been recognized for over 25 years that pyrethroid insecticides are hydrolyzed by esterases and oxidized by CYP450s. Tetraethyl pyrophosphate and  $S,S,S$ -tributyl phosphorotrithionate (specific esterase inhibitors) and piperonyl butoxide (a CYP450 inhibitor) increased the acute toxicity and decreased the metabolism of pyrethroid insecticides (Ruzo et al., 1979; Shono et al., 1979). There has been, however, a quite limited amount of information on the identity and characteristics of the specific enzymes responsible for biotransformation of pyrethroid insecti-

cides. Different sensitivities to inhibition by organophosphates led Soderlund et al. (1982) to conclude that several esterases hydrolyzed pyrethroid insecticides in mouse and rat liver microsomes. Stok et al. (2004) have recently isolated and characterized two carboxylesterases from mouse liver that metabolize pyrethroid compounds to varying degrees.

Satoh et al. (1999) proposed that A-esterases were involved in metabolism of pyrethroid insecticides but did not provide any supporting data. We report here that plasma (Fig. 4) and liver (data not shown) A-esterases are apparently not involved in the hydrolysis of deltamethrin. The experiments with purified horse serum butyrylcholinesterase (data not shown) preclude the involvement of butyrylcholinesterase in the metabolism of deltamethrin.

To characterize deltamethrin metabolism by carboxylesterases, the efficacy of PSP and isoOMPA, known inhibitors of carboxylesterases, was examined in the current study. Interestingly, both PSP and isoOMPA inhibited 90% of carboxylesterase activity in liver microsomes, but only 50% of carboxylesterase activity in plasma (Fig. 2). Although it is well established that carboxylesterases are synthesized in liver and secreted into the bloodstream (Alexson et al., 1994; Yan et al., 1995), their differential sensitivity to PSP or isoOMPA could be due to the glycosylation of the plasma enzymes (Yan et al., 1995) and/or minor differences in protein sequence (Alexson et al., 1994).

Rat plasma contains relatively high levels of carboxylesterase activity and therefore serves as a site of deltamethrin biotransformation. The high Vmax and Km, however, determined in the current study indicate that deltamethrin is metabolized by high-capacity, low-affinity enzyme(s) in rat plasma. The nature of the isoOMPA-insensitive metabolism of deltamethrin in the plasma is unclear. It is possible that hydrolytic cleavage of deltamethrin at higher concentrations (100 and 200  $\mu\text{M}$ ) is by isoOMPA-insensitive carboxylesterase isoforms because: (1) A-esterase and butyrylcholinesterases are not responsible (Fig. 4); (2) in the microtiter plate reader assay, 1 mM isoOMPA inhibited only 50% of the hydrolysis of pNPA (Fig. 2); and (3) Chambers et al. (1991) reported that rat serum carboxylesterases show differing substrate specificities and inhibitor sensitivities. It may also be possible that another enzyme (or a protein that has esterase activity)

contributes to deltamethrin metabolism at higher concentrations. It is, however, noteworthy that deltamethrin concentrations (2 and 20  $\mu\text{M}$ ) at which metabolism was completely inhibited by isoOMPA are only slightly higher than peak blood levels in severely poisoned rats (Anàdon et al., 1996; Haines et al., 2004). Thus, the rate constants ( $V_{\text{max}} = 325.3 \pm 53.4$  nmol/h/ml and  $K_m = 165.4 \pm 41.9$   $\mu\text{M}$ ) for isoOMPA-sensitive metabolism in plasma are applicable to non-lethal exposure conditions in vivo. These rate constants were considered as carboxylesterase metabolism. Since isoOMPA inhibits metabolism at realistic concentrations in vivo, we did not attempt to address the identity of isoOMPA-insensitive metabolism.

Biotransformation of deltamethrin is markedly greater in the liver than in plasma. Intrinsic clearance by liver carboxylesterases was higher than that by plasma carboxylesterases (Table 1). Our finding of a higher  $V_{\text{max}}$  for liver carboxylesterases, in conjunction with the similar affinity of the liver and plasma enzymes, is consistent with greater carboxylesterase activity in the rat's liver (Chanda et al., 1997; Satoh and Hosokawa, 1998). Another possible reason for the differences in the  $V_{\text{max}}$  values may be variations in structure and protein sequence of these enzymes (Alexson et al., 1994; Yan et al., 1995). Carboxylesterases are a family of enzymes that manifest different sensitivities to inhibitors (Chambers et al., 1991; Jekanoviæ et al., 1996; Soderlund et al., 1982). Although liver carboxylesterases hydrolyze deltamethrin more efficiently than do plasma carboxylesterases, hepatic deltamethrin metabolism is due largely to CYP450s. Our results show that intrinsic clearance of deltamethrin by hepatic CYP450s is considerably higher than by hepatic or plasma carboxylesterases (Table 1). This is attributable to CYP450s' higher affinity and higher metabolic capacity. Animal studies support the considerable contribution of hepatic CYP450s to deltamethrin metabolism. Pretreatment of mice with tributyl phosphorotrithioate, an esterase inhibitor, results in a 2-fold decrease in deltamethrin's  $\text{LD}_{50}$  (Ruzo et al., 1979). Pretreatment with piperonyl butoxide, a CYP450s inhibitor, produces an even greater decrease in the  $\text{LD}_{50}$  (i.e., 3- to 5-fold) (Ruzo et al., 1979). Both treatments reduced urinary excretion of deltamethrin metabolites and enhanced brain levels of deltamethrin. In addition, when [ $^{14}\text{C}$ ]-labeled deltamethrin (1  $\mu\text{g}$ ) was incubated with mouse liver microsomes, contribution from oxidases (most likely CYP450s) was higher (41%) than esterases (28.3%) to total deltamethrin metabolism (Shono et al., 1979).

The ability of carboxylesterases and CYP450s to metabolize pyrethroid insecticides appears to be dependent largely upon the compounds' structure. Soderlund and Casida (1977) observed that the *trans* isomer of permethrin was much more susceptible to hydrolysis by esterases than the *cis* isomer. Similar findings were reported with rat liver microsomes (Shono et al., 1979) and isolated carboxylesterases (Stok et al., 2004). In a study of 44 pyrethroids, Soderlund and Casida (1977) observed that those with a

*cyano* group were the least sensitive to hydrolysis and less prone to oxidation. Deltamethrin is present solely as the *cis* isomer, contains a *cyano* group (Fig. 1), and was designed for maximum duration of action and insecticidal potency. Deltamethrin's resistance to metabolism is reflected by its long half-life in rats, relative to permethrin, a non-*cyano* pyrethroid (Anàdon et al., 1991, 1996).

Studies using rat Supersomes suggest that CYP1A1, CYP1A2, and CYP2C11 are capable of metabolizing deltamethrin, with CYP2C11 showing the least activity (Fig. 7). Although there is no direct evidence, Dayal et al. (1999, 2003) implicated CYP2B1/2 and CYP1A1/2 in deltamethrin metabolism from their observation that the subacute deltamethrin administration induced hepatic CYP1A1/2 and CYP2B1/2 activities and inducers of these isozymes altered the toxicity in rats. Deltamethrin was not metabolized by CYP2B1 in the system we utilized, but it may be oxidized by CYP2B2 or other isoforms that are not commercially available at present.

In summary, carboxylesterases appear to be the esterases responsible for deltamethrin metabolism in rat liver microsomes; carboxylesterases account for a portion of deltamethrin metabolism in plasma. Liver carboxylesterases are more efficient than the plasma carboxylesterases in the metabolism of deltamethrin, whereas CYP450-catalyzed deltamethrin metabolism is greater than that by either plasma or liver carboxylesterases. The relatively low intrinsic clearance of deltamethrin by plasma carboxylesterases indicates that detoxification in the bloodstream is likely to be very limited compared to that in the liver. If it is assumed: (a) a 400-g rat's blood and liver volume/weight are 28 ml and 15 g, respectively (Brown et al., 1997); (b) the plasma  $V_{\text{max}}$  at relevant deltamethrin levels is 325 nmol/h/ml (Table 1); and (c) the liver  $V_{\text{max}}$  is 4592 nmol/h/g (i.e., the sum of the liver carboxylesterases and CYP450  $V_{\text{max}}$  values) (Table 1); then it can be estimated that the maximum amount of deltamethrin that can be metabolized per hour by the plasma is 9.1  $\mu\text{mol}$ , but liver clearance would be approximately 8 times higher at 68.9  $\mu\text{mol}$ . This information on major sites of metabolism, the relative importance of detoxification pathways, and their associated rate constants is being utilized to develop a PBPK model to forecast internal dosimetry and toxicity of deltamethrin in the adult rat.

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signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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